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Research Article

Practical Application and Influence of "Avigen duck" Immunomodulator to White Pekin Ducks Humoral Immune Factors

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Abstract: Factors of non-specific immunity are responsible for protecting birds from a number of viruses and bacteria. Bactericidal proteins are played here lysozyme and complement, interferon, as well as beta-lysine. Adding appropriate active compounds to the bird's diet to activate the innate immune response are subject to studies by many authors. The practical application and influence of the "Avigen Duck". immunomodulator in the cultivation of White Pekin ducks aims to clarify the dynamics of the indicators. It was found that the ducks treated with the immunomodulator had higher non-specific protection expressed in the values of the observed indicators. Complement activity for the experienced group (529.45 ± 17,85 CH50) is significantly higher than that of untrained birds (308.56 ± 10.19 CH50), on the 30th day of their lives (P <0.001). Lysozyme values for the experienced group (6.34 ± 0.86 mg L^{-1}) is more than twice as high as its concentration in control birds - 2.52 ± 0.59 mg L^{-1}. At 30 days of age, the mean concentration of IFN γ in the control group of ducks was 108.86±6.12 pg ml^{-1}, and in the experimental group treated with immunomodulator - 518.06±12.80 pg ml^{-1}, accompanied by an increase in the values of IL-2 and IL-6 and beta-lysine activity. These data show that, despite the short life of the ducks, the concentrations of the investigated factors of non-specific immunity increase significantly and statistically reliably, even after a single treatment with immunomodulator "Avigen Duck". The practical application of the immunomodulator in the duck diet has a strong effect on their non-specific immunity.

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1. Introduction

Modern poultry farming requires fast-growing periods, high food conversion ratios, and maintenance of health status at the lowest possible cost. One of the biggest challenges is the high density of the bird population, raised in relatively small spaces, which increases the risk of spontaneous disease outbreaks (Linares and Martin, 2010). If the first two necessities are influenced by the nutritional

properties of the food provided. The third requirement has a far more complex nature where animal selection and the introduction of specific food supplements play a crucial role. However, targeting a high immune response against a specific pathogen is not always the best option. In several cases, farmers face subclinical infections from a well-known cocktail of locally presented infectious agents. This category of diseases has a massive impact on animals' productivity and well-being. The unpredictable nature of such processes requires prominent levels of non-specific immune response factors among all animals (Al-Mansour et al., 2011).

Two major factors of natural humoral immunity are the complement activity and the lysozyme values in the blood serum. The complement system and the two major pathways of its activation are one of the oldest mechanisms for protection against pathogenic bacteria and play a crucial role in both specific and nonspecific immune responses. The system consists of several proteins, produced in an inactive form (Karakolev, 2014). Its activation results in a cascade of consecutive reactions aimed at eliminating a specific infectious agent. The activation of the complement in the absence of a specific antigen is an alternative variant of classic activation and does not require the formation of an antigen-antibody complex. This makes it one of the very first defense mechanisms against a wide range of pathogens. Its key role remains further evidenced by the fact that this mechanism was the first to evolve in evolution (Zewde et al., 2016). The triggering of the alternative mechanism complement system is quite effective against different gram-negative bacteria, viruses, neoplastic cells, etc. (Cortes et al., 2013). Lysozyme is an antimicrobial enzyme that has essential functions in the innate immune response. Muramidase is found in egg white, saliva, blood serum, etc. The serum lysozyme is predominantly produced by the macrophages (Besarabov, 2013) and is effective against Gram-negative bacteria as well as some large viruses, such as *Avipoxvirus* (Zhang et al., 2017). Breed, sex, age, and species variations in both parameters have been observed (Koynarski et al., 2018; Koynaski and Sotirov, 2013). Given the large number of genes encoding different protein fractions for both parameters of interest, marker-assisted selection does not provide a useful solution. Moreover, poultry farming has always focused more on productive parameters than health and welfare (Bahmanimehr, 2012). In the past, fighting pathogens favored using antibiotics, including some with nutritional effects. Nowadays trends for the production of antibiotic-free animal products motivate the use of different probiotics, symbiotics, immunostimulants, and vaccines (Jiang et al., 2015; Wang et al., 2016). Many of these substances have pathogen inhibition, growth performance, and welfare properties (Chen et al., 2017). At present, the market offers several immunomodulators of herbal origin, but their effect is limited (Georgieva et al., 2013). Given that triggering molecules for APCA and lysozyme actions are lipopolysaccharides found in the bacterial cell membrane and viral envelopes, using an immunomodulator based on the same concept seems promising. Beta-lysine is part of the thermostable bactericidal fractions in serum active against bacilli that do not require the presence of complement to perform their function (Weinert, 2013). Their influence affects both non-specific resistance and homeostasis. Interferon γ are products of T-lymphocytes and natural killer cells in birds and participate in all immune reactions, and IFN α/β are mainly responsible for carrying out antiviral immunity (Masuda et al., 2012, Karakolev, 2014, Swaggerty et al. 2015). Bacterial lipopolysaccharides are known to be a powerful inductor of interferon (Lalev et al., 2015). IL-2 and IL-6 in birds play an important role as regulation factors and mediators of the immune response (Fernando et al., 2015).

The major focus of this study was given to the impact of the "Avigen Duck" on some innate immune factors among White Pekin ducks. Since the product is based on lipopolysaccharide components of the thermostable endotoxin of Gram-negative bacteria from *Enterobacteriaceae* we could assume its stimulating effect on parameters of the innate immune response.

2. Material and Methods

2.1. Birds

White Pekin ducks of two flocks - experienced and controlled, grown under the same production conditions. The birds of the experienced flock received an immunomodulator from the 1st to the 10th day of the fattening period. The birds of the control flock did not receive an immunomodulator. Both flocks were treated with the preparations "Aspivit C" and "Bioxan", which played an auxiliary role. The first has an anti-stress effect, and the second increases the permeability of the mucous intestinal surfaces.

2.2. Method of treatment

Polybacterial immunomodulator "Avigen Duck" is a concentrated form of lipopolysaccharide, extracted from the Enterobacteriaceae family. The immunomodulator was administered in liquid form, containing 3 000 doses (ten days) in 1000 ml. It is used orally through drinking water, using the available farm dispensers.

2.3. Sampling

The experimental and control flocks numbered 14 000 birds each. From the birds treated with the immunomodulator in the experimental flock, as well as from the control one, 45 birds were randomly selected, from which blood was taken for testing. On the 20th and 30th day of the life of the birds (the 10th and 20th day after treatment with the immunomodulator) we took blood from the axillary vein. The separated blood serum was stored at 4°C. Serum testing was not later than 24 hours after sampling.

2.4. Determination of interferon-γ, interleukin-2 and interleukin-6

The interferon concentration was determined by enzyme-linked immunosorbent assay. We used DUCK Interferon (IFN γ) ELISA kit, DUCK Interleukin ELISA kit (MYBIOSOURCE). We added the following standards to the wells of the plate with concentrations: 0, 15.6, 31.2, 62.5, 125, 250, 500, and 1000 pg ml^{-1} for IFN γ, for IL-2 we prepared standards of 0, 6.25, 12.5, 25, 50, 100, 200 and 400 pg ml^{-1}, and for IL-6 - 6.25, 12.5, 25, 50, 100, 200 pg ml^{-1}, respectively. Extinctions were measured at a wavelength of 450 nm. We have calculated concentrations based on a standard curve.

2.5. Determination of complement activity and lysozyme concentrations

Complement activation was evaluated by the method of (Sotirov et al., 2005). One hundred microliters of each serum sample were diluted with 350 µl veronal-veronal Na buffer (in final concentrations: 146 mM NaCI, 1.8 mM 5.5-diethylbarbi-turic acid sodium salt; 3.2 mM 5.5-diethylbarbituric acid; 1 mM EGTA and 0.8 mM $MgCl_2$). Using U bottomed plates, 7 other dilutions from each diluted serum were again prepared in veronal-veronal Na buffer, so the final serum dilutions were 8/45, 7/45, 6/45, 5/45, 4/45, 3/45, and 2/45, respectively. Subsequently, each well was supplemented with 100 µl of 1% rabbit erythrocyte suspension. Samples were incubated for 1 hour at 37°C statically and then centrifuged at 150 g for 3 minutes at room temperature. Thereafter, 150 µl of each supernatant was placed into a flat-bottomed plate for measurement of optical density at 540 nm using 'Sumal-PE2' ELISA reader (Karl Zeiss, Germany). The final APCA activity was calculated using a dedicated software developed at Trakia University (Bulgaria) and expressed as CH50 units (corresponding to 50% of complement-induced hemolysis of applied erythrocytes).

Blood serum lysozyme concentrations were analyzed by the method of (Sotirov and Koynarski, 2003). The method consists of mixing 20 ml of 2% agarose dissolved in phosphate buffer (0.07 M $NaHPO_4$ and NaH_2PO_4) with 20 ml suspension of a 24-hour culture of *Micrococcus lysodeicticus* at 67°C. While still warm the mixture is poured into a 14-cm Petri dish. After solidifying at room temperature, 5 mm wells are made. Each well is filled with 50 µl of undiluted serum. Eight standard lysozyme dilutions (from 0.025 to 3.125 µg/ml) are prepared and pipetted into eight wells. The plate is then incubated for 20 hours at 37°C. The final lysozyme concentration is calculated by dedicated software developed at Trakia University (Bulgaria), comparing the lytic zone of each sample with the standard lysozyme dilutions.

2.6. Determination of beta-lysine activity.

The beta-lysine activity of blood serum was determined by a spectrophotometric method of Bucharin et al. (1977), modified by Karakolev and Nikolov (2015). The research was performed in flat-bottomed plates. We used a pre-prepared spore suspension of Bacillus subtilis ATCC 6633. We added the controls with an automatic pipette - 80 µl of saline + 80 µl of spore suspension in each of the first 4 wells. We then instilled the experimental sera with an automatic pipette - 80 µl serum + 80 µl spore suspension in each of the following wells, according to the number of samples tested. We homogenized it with a plate shaker. Optical density measurements were performed using a BioTek L80 spectrophotometer at a wavelength of 630 nm, before incubation. We incubated the plate in a plate

incubator with a timer (37°C for 2 hours). Immediately after incubation, we again measured the optical densities at the same wavelength. Since the optical densities of the controls did not change for 2 hours in the incubator, we performed the calculations by taking the changes in the optical densities of the samples, for each well separately, according to the formula:

$$\% \text{ of lysis} = OD1 - OD2 / OD1 \times 100 \quad (1)$$

where OD1 is the optical density of the sample before incubation and OD2 is the optical density of the sample after incubation.

2.7. Statistical analysis

Obtained data have been processed by independent t test with the fixe defect model using the Data analysis tool pack, Microsoft Excel 2016, Microsoft Corporation Ltd., at a level of significance $P < 0.001$.

3. Results

3.1. Values of lysozyme, complement and beta-lysine activity in blood serum

As can be seen from the Table. 1 the lysozyme values in experimental birds was higher than in the control flock ($P<0.001$). Lysozyme concentrations varied from 6.58 mg L^{-1} to 6.34 mg L^{-1} in experimental ducks. In untreated birds, these levels were significantly lower and ranged from 3.08 mg L^{-1} to 2.52 mg L^{-1}. In experimental flock, significantly higher APCA activity (529.45 CH 50) was found while in control chickens this value was lower (308.56CH50; $P <0.001$) on the 30th day of the life.

Table.1. Serum lysozyme concentrations, complement activity (APCA) and beta-lysine activity.

Groups	Lysozyme (mg mL^{-1})	APCA (CH50)	Beta-lysine activity (%)
White Pekin ducks, 20th day of the life			
Experimental	6.58±0.94***	506.52±18.34***	32.58±1.55***
Control	3.08±0.68	311.40±15.50	12.52±1.40
White Pekin ducks, 30th day of the life			
Experimental	6.34±0.86***	529.45±17.85***	34.89±0.95***
Control	2.52±0.59	308.56±10.19	12.60±1.72

*** $P < 0.001$.

Beta-lysine activity in blood serum was also higher than measured in birds untreated with the immunomodulator – from 32.58% to 34.89% in experimental ducks against from 12.52% to 12.60% in the control flock ($P<0.001$).

3.2. Values of interferon-γ, interleukin-2 and interleukin-6 in blood serum

Interferon γ and interleukins concentrations in birds are presented in Table 2. In birds treated with "AVIGEN DUCK", significantly higher concentrations of IFN γ were found compared to the control flock ($P <0.001$). These values fluctuate between 540.45 pg mL^{-1} and 518.06 pg mL^{-1}, and although they slightly decrease by day 30, remain much higher than the concentrations of controls.

The concentration of interleukins also responded in a positive direction as a result of the administration of the immunomodulator. In control birds, the levels of IL-2 hesitated between 6.30 pg mL^{-1} and 6.54 pg mL^{-1}, respectively on the 20th and 30th day of the life of the birds, while in the experimental group, there was an increase in these values. We have received similar data for the levels of IL-6. In birds treated with "Avigen Duck", the concentration of IL-6 in the blood serum reached 95.10 pg mL^{-1}, while in the control groups, it remained at low values – from 8.65 to 9.22 pg mL^{-1}.

Table 2. Concentrations of interferon-γ, interleukin-2 and interleukin-6 in blood serum

Groups	IFN γ (pg mL^{-1})	IL-2 (pg mL^{-1})	IL-6 (pg mL^{-1})
White Pekin ducks, 20th day of the life			
Experimental	540.45±10.20***	10.46±0.58***	70.25±7.40***
Control	119.22±6.58	6.30±0.90	8.65±2.72
White Pekin ducks, 30th day of the life			
Experimental	518.06±12.80***	10.98±0.66***	95.10±8.19***
Control	108.86±6.12	6.54±0.75	9.22±2.50

*** $P < 0.001$.

4. Discussion

In all our tests in blood serum, we found a higher concentration of lysozyme, higher complement activity and beta-lysine activity in birds from the experimental flock that received an immunomodulator. Other non-specific humoral indicators - interferon γ, interleukins 2 and 6, also showed increased values in the experienced flock, compared to the control. These indicators are likely to respond of stimulating mucosal membranes with lipopolysaccharides of polybacterial immunomodulator, as well as the auxiliary action of the preparations "Aspivit C" and "Bioxan", with the latter helping to improve the superficial absorption of the intestinal mucosa. In addition, the application of the anti-stress preparation "ASPIVIT C" provides additional equalization in the conditions of the experiments that were conducted under production conditions. This makes it possible to exclude the influence of some technological stressors and to believe with a high degree of confidence that the increase in lysozyme, complement, beta-lysine activity, IFN γ, IL 2, and IL 6 in experimental birds is due to the effect of the immunomodulator "AVIGEN DUCK". In any case, the results obtained from the control herds and reflected in Tables 1 and 2 confirm this judgment. Several studies on germ-free animals show that symbiotic bacteria and/or bacterial molecules (lipopolysaccharides, β-glucan and peptidoglycans) can completely cause a non-specific immune response (Gensollen et al., 2016; Ganalvonarburg et al., 2016; MacPherson et al., 2017), as the intestinal mucosa has its leading meaning in the initial activation of the innate immunity and influence its regulation and maturation.

Lipopolysaccharides of polybacterial immunomodulators are powerful inductors of lysozyme, complement, interferon, and cytokines (Karakolev et al., 2014). The current experiments also establish their effect on beta-shade activity, which is part of the non-specific immune factors in the blood serum. Obviously, lipopolysaccharides are one of the earliest factors stimulating congenital immunity, and their additional activity can be induced by lipopolysaccharides by enterobacteria included in the drinking water of birds, as our experiments show. According to Cellak and Babacanoglu (2022) on the blood biochemical parameters of broiler chicks, can be influenced very early by the injection of leptin in the yolk sac of the embryo.

In our previous studies, the physiological values of beta-lysine in the blood serum of broiler breeders and broilers were monitored. Unlike broilers, in parent flocks, there is a strong stress factor associated with the onset of laying and accompanying hormonal and immune rearrangement in the body (Karakolev, 2015; Karakolev and Nikolov, 2015). Nevertheless, the concentration of beta-lysine may be influenced by some factors that have a beneficial effect on the activity of complement, lysozyme and other indicators of the natural (innate) immune response. A number of authors (Ganalvonarburg et. al., 2016; Zemskov et al., 2018; Bozakova et al., 2018) consider the factors that may influence the mechanisms of nonspecific, innate resistance and emphasize that polysaccharide substances are one of the most effective for this purpose. Hung et al. (2013) consider that AblAs from methanoarchaea are lysine 2,3-aminomutases that may function as potential biocatalysts for the synthesis of β-lysine *in vivo* and *in vitro*. Okanishi et al. (2013), Zhang et al. (2013), Weinert et al. (2013), also developed genetic methods for the biosynthesis of lysine for biotechnological purposes. From the present experiments, it is clear that lipopolysaccharides from enterobacteria contained in concentrated form in the immunomodulator "AVIGEN DUCK" have a positive effect on the activity of beta-lysin fractions in the blood serum as well as other observed indicators. Increased activity of IFN and IL-4, IL-6, and IL-15 monitors Liao et al. (2021) after the treatment of Muskovy ducklings with *Astragalus polysaccharide*

(APS). The results showed that APS significantly affects intestinal injuries of villi length and wall thickness of the small intestine infected with Muscovy duck reovirus, subsequently increasing sIgA and all the cytokine productions at most time points, suggesting that APS pretreatment can effectively stimulate mucosal immune function by improving intestinal morphology. Similar data in broilers reported Sotirov et al. (2021), which compare the effect of the application of *Schizochytrium limacinum* on some indicators of natural immunity in broilers. The authors found an increase in the activity of beta-lysine in the blood serum in experimental group III, accompanied by a slight decrease in the values of lysozyme and complement. Bozakova et al., (2018) followed the influence of the immunomodulator "Immunobeta" on innate humoral immunity in laying hens and also found changes in beta-lysine activity. In conditions of temperature stress (Bozakova et al., 2018) studied the effect of the immunomodulator "Immunobeta" on innate humoral immunity in turkeys and laying hens, which supports our data on the special role of beta-lysine in stressful situations. The results we have obtained are in line with the established by other modern authors about the ability of polysaccharides of different origins, including vegetables, to regulate the immune response in fattening ducks and broiler chickens (Liu et al., 2016; Wang et al., 2016; Li et al., 2018).

Following the administration of the "AVIGEN DUCK" immunomodulator, the activity of nonspecific immune factors in the blood serum of White Pekin ducks increases well above the physiological boundary observed in the control flock grown under the same conditions. In this way, the nonspecific resistance of ducks, which is of a lasting character, is of particular importance for their health status.

Conclusion

The experimental data we receive, allow the conclusion to be made that the „Avigen Duck" immunomodulator containing lipopolysaccharides of enterobacteria and administered with drinking water at a suitable dose causes an increase in nonspecific resistance factors in White Pekin ducks and is suitable for use, in view of their health status when growing for meat.

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